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Barley Stem Rust Resistance Genes: Structure and Function

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Abstract

Rusts are biotrophic pathogens that attack many plant species but are particularly destructive on cereal crops. The stem rusts (caused by *Puccinia graminis*) have historically caused severe crop losses and continue to threaten production today. Barley (*Hordeum vulgare* L.) breeders have controlled major stem rust epidemics since the 1940s with a single durable resistance gene *Rpg1*. As new epidemics have threatened, additional resistance genes were identified to counter new rust races, such as the *rpg4/Rpg5* complex locus against races QCCJ and TTKSK. To understand how these genes work, we initiated research to clone and characterize them. The *Rpg1* gene encodes a unique protein kinase with dual kinase domains, an active kinase, and a pseudokinase. Function of both domains is essential to confer resistance. The *rpg4* and *Rpg5* genes are closely linked and function coordinately to confer resistance to several wheat (*Triticum aestivum* L.) stem rust races, including the race TTKSK (also called Ug99) that threatens the world's barley and wheat crops. The *Rpg5* gene encodes typical resistance gene domains NBS, LRR, and protein kinase but is unique in that all three domains reside in a single gene, a previously unknown structure among plant disease resistance genes. The *rpg4* gene encodes an actin depolymerizing factor that functions in cytoskeleton rearrangement.

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Rusts are biotrophic fungal pathogens (phylum: Basidiomycota) that cause disease on almost every major family of plants (Agrios, 2005). *Puccinia* is by far the largest genus of rust fungi with more than 5000 described species (Cummins and Hiratsuka, 2003; Swann et al., 2001). The most economically significant *Puccinia* species are those that attack the cereal crops of wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), oat (*Avena sativa* L.), rye (*Secale* L.), and maize (*Zea mays* L.). The cereal rusts, especially those attacking the major food crops such as wheat and barley, have caused famines throughout history, and epidemics have been

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Abbreviations: aa, amino acid; ADF, actin depolymerizing factor; Avr, avirulence; eQTL, expression quantitative trait loci; HR, hypersensitive response; *Hv*RGA, *Hordeum vulgare* resistance gene analog; JAK, Janus kinase; LRR, leucine rich repeat; NBS, nucleotide binding site; *Pgt*, *Puccinia graminis* f. sp. *tritici*; QTL, quantitative trait locus; *R*-genes, resistance genes; R-proteins, resistance proteins; RAPD, random amplified polymorphic DNA; RFLP, restriction fragment length polymorphism; S/TPK, serine/threonine protein kinase; STAT; signal transducer and activator of transcription; TIR, toll Interleukin receptor; Ug99, Uganda 1999; VIGS, virus induced gene silencing.

documented in the literature since the time of Aristotle (Bushnell and Roelfs, 1984).

Wheat and barley are hosts to stem, leaf, and stripe rusts. The stem rust pathogen *Puccinia graminis* is composed of a number of different *formae speciales* or "special forms" that principally attack one or a few hosts. For example, *Puccinia graminis* f. sp. *tritici* attacks primarily wheat but also barley and several grass hosts; *P. graminis* f. sp. *secalis* attacks primarily rye but also barley and several grass hosts; and *P. graminis* f. sp. *avenae* attacks primarily oat but other grasses as well (Farr et al., 1995). *Puccinia triticina* (formerly *P. recondita* f. sp. *tritici*) and *P. hordei* cause leaf rust or brown rust on wheat and barley, respectively. *Puccinia striiformis* f. sp. *tritici* and *P. striiformis* f. sp. *hordei* cause stripe rust or yellow rust on wheat and barley, respectively (Farr et al., 1995).

Deployment of rust resistant cultivars has been the primary strategy for combating the rusts (Kolmer, 2001; Steffenson, 1992). The release of wheat cultivars with single major resistance genes led to the "boom-and-bust" cycles of wheat breeding, culminating in the catastrophic stem rust epidemics of the 1950s where more than 11 million t (400 million bushels) of wheat were lost in the Great Plains. Since the 1960s, incorporating multiple resistance genes into cultivars has effectively controlled stem rust of wheat. In contrast, barley stem rust has been kept in check since the 1940s by breeding cultivars with one major durable gene, *Rpg1*; however, other factors such as a largely resistant wheat crop and barley's shorter maturation period may contribute to the long-lasting disease control (Steffenson, 1992). The rusts remain a continued threat to cereal production because they are extremely variable with regard to virulence (Leonard, 2001), have explosive reproductive capacity, and can readily spread over long distances on the wind. Indeed, a new race of *P. graminis* f. sp. *tritici* (QCCJ) appeared in 1989 and caused some losses of barley and wheat in the Great Plains (Roelfs et al., 1993). Another threat to wheat and barley worldwide is TTKSK (also called Ug99), a widely virulent race that was first discovered in Uganda in 1998 (Pretorius et al., 2000) and has since spread throughout East Africa (Singh et al., 2008) and recently to the Middle East (Nazari et al., 2009). Although TTKSK has not spread to North America yet, it is the most serious threat to cereal production in more than 50 yr because it is virulent to most wheat and barley cultivars grown in the major wheat- and barley-producing regions worldwide (Singh et al., 2008; Raloff, 2005).

Puccinia g. f. sp. *tritici* is one of the most intensively studied fungal plant pathogen systems (Roelfs, 1985). After Eriksson forwarded the concept of the *formae speciales* of *P. graminis* (Agrios, 2005), Stakman reported an even finer level of host specialization: the ability of *P. graminis* f. sp. *tritici* isolates to specifically attack just some wheat genotypes and not others. This concept of physiological specialization in rust fungi led to the classification of races of *P. graminis* f. sp. *tritici* and development of wheat differentials to identify them (Stakman and Piemeisel, 1917).

The most economical means of controlling stem rust is by incorporation of resistance genes into cultivars (Kolmer, 2001; Steffenson, 1992). Currently, more than 40 stem rust resistance genes have been identified in wheat (McIntosh et al., 1995). In barley, seven genes conferring stem rust resistance are recognized, but only *Rpg1* has been widely utilized in commercial cultivars.

An ultrastructural karyotype of *P. graminis* f. sp. *tritici* was completed by tracing and reconstructing serial sections from pachytene nuclei from electron micrographs. The number of haploid chromosomes found was 18, and they ranged in size from 3.0 to 8.8% of the total cytological length of the genome (Boehm et al., 1992). Early studies of genome size done by reassociation kinetics estimated it to be 67Mb (Backlund and Szabo, 1993). The fungus was recently sequenced and assembly to date suggests a genome size of 88.64 Mb with 20,567 predicted genes, which is likely to be an underestimation (*Puccinia graminis* f. sp. *tritici* Database, http://www.broad.mit.edu/annotation/genome/puccinia_graminis/).

The most extensively investigated rust resistance system is in flax (*Linum perenne* L.). Five multiple-allele loci (*L, M, P, N*, and *K*) have been identified (Ellis et al., 1988), and four (*L, M, P* and *N*) have been cloned (Anderson et al., 1997; Dodds et al., 2001; Lawrence et al., 1995). All four genes are of the TIR-NBS-LRR type. The *L* and *M* genes are closely related and probably represent homeologous loci since flax is an ancient tetraploid. The *P* and *N* loci consist of small multigene families (Dodds et al., 2001). The LRR region was shown to be a determinant of specificity differences between *L* alleles (Ayliffe et al., 1999). However, the TIR region may also be involved (Ellis et al., 1999; Luck et al., 2000). Truncation of the *P2* C-terminal non-LRR domain caused loss of function, suggesting another region for specificity determination (Dodds et al., 2001). A G to E amino acid substitution in the P2 GLPL domain motif resulted in loss of function, providing direct evidence for the role of this highly conserved motif in plant disease resistance (Dodds et al., 2001). The first rust *Avr* gene was cloned from flax rust (*Melampsora lini*) by map-based cloning (Dodds et al., 2004). This *AvrL567* gene, shown to be expressed in the haustoria, encodes a 127 amino acid (aa) secreted protein and is delivered to the host cells across the plant membrane (Dodds et al., 2004). This knowledge was exploited to isolate additional flax rust *Avr* genes (Catanzariti et al., 2006). Interestingly, the flax rust Avr567 protein interacts directly with the corresponding L5, L6 and L7 R proteins in vivo and in yeast two-hybrid assay (Dodds et al., 2006).

In cereals, two thoroughly investigated rust resistance systems are the maize *Rp1* and *Rp3* genes conferring resistance to common rust (*P. sorghi*) and the barley stem rust resistance genes *Rpg1*, *rpg4* and *Rpg5*. *Rp1* and *Rp3* of maize are complex loci consisting of clusters of closely related NBS-LRR genes (Collins et al., 1999; Webb et al., 2003). Their tendency to mispair and recombine in meiosis creates haplotypes that are structurally variable. For example, *Rp1* haplotypes in different maize

lines vary in the number of *Rp1* genes they carry from a single gene to more than 50 (Smith et al., 2004). Many recombinant genes and haplotypes have been identified that have interesting phenotypes (Hulbert, 1997). In addition to typical race-specific resistance genes like *Rp1-D,* recombinant genes have been identified that give an extensive hypersensitive reaction to inoculation with any rust isolate, including typically noncongenial species like the wheat rusts (Hu et al., 1996). These also confer lesion mimic phenotypes unless grown aseptically, presumably due to triggering of HR by rust spores and possibly other microbes. The best characterized of these nonspecific resistance genes is *Rp1-D21*, which was derived from a recombination event between the *Rp1-D* and another *rp1* gene that had no known phenotype.

Several *Rp1* haplotypes have been identified that provide quantitative levels of rust resistance at the adult plant stage (Hu et al., 1997). This resistance appears to be nonspecific and confers moderate resistance to both *P. sorghi* and tropical maize rust (*P. polysora*). This resistance is similar to that reported for *Lr34* and *Lr46*, two wheat genes that confer adult plant leaf rust resistance and are popular in breeding programs due to their durability (Kolmer, 1996; Martinez et al., 2001). These wheat genes are also thought to confer adult-plant resistance to other rust species besides leaf rust (McIntosh, 1992; Singh, 1992). Other interesting parallels between the *rp1* haplotypes and the wheat genes are that both are associated with adult-plant phenotypes that can be observed without rust inoculation in the proper environment and both are very dependent on genetic background (German and Kolmer, 1992). The phenotypes are leaf-tip necrosis in wheat and chlorotic–necrotic flecking on the adult leaves of maize (Hu et al., 1997). In maize, genetic backgrounds have been identified that inhibit these adult-plant effects and are severe in others depending on the genetic background. The barley stem rust resistance genes are discussed in detail below.

Physical interaction between resistance (R) and avirulence (AVR) proteins has been demonstrated for a few of the *R* genes for which it has been examined, i.e., *Pto*, *Pi-ta*, *Rrs1,* and *L6* (Deslandes et al., 2003; Jia et al., 2000; Tang et al., 1996). The failure to detect physical interaction between R and AVR proteins led to the "guard hypothesis," which postulates that most R proteins recognize the interaction between AVR proteins and their respective virulence target proteins. For example, the PRF protein recognizes the interaction between PTO and AVRPTO and activates a defense response (Van Der Bizen and Jones, 1998). The guard hypothesis implies that each NBS-LRR type R protein should have a target protein that it guards. Interactions where additional proteins, besides those encoded by the *R* and *Avr* genes, are required for the resistance reaction also support a guard model (Dixon et al., 2000; Ren et al., 2000; Swiderski and Innes, 2001).

The guard hypothesis also predicts that the R protein could respond to more than one AVR protein, as is demonstrated with PTO and RPM1. RPM1 is an *Arabidopsis* NBS-LRR protein that confers resistance to *Pseudomonas syringae* expressing either *AvrRpm1* or *AvrB* genes that are unrelated at the sequence level (Grant et al., 1995). Each AVR protein interacts with and phosphorylates the *Arabidopsis* RIN4 protein (Mackey et al., 2002). Thus, RIN4 may be the target of both avirulence gene products and RPM1 may act to guard RIN4. The avirulence gene target may be guarded by more than one R protein as demonstrated for the *Arabidopsis* RIN4 protein. RPS2 is an NBS-LRR protein that induces resistance against *P. syringae* expressing the *AvrRpt2* gene (Bent et al., 1994; Mindrinos et al., 1994). AVRRPT2 also targets RIN4, causing its disappearance at the post-transcriptional level. This activity appears to be independent of RPS2 function (Mackey et al., 2003). Thus, the simple receptor-ligand gene for gene model must be extended to include a coreceptor and/or guard model.

Barley Stem Rust Resistance Genes

Seven genes conferring resistance to *P. graminis* f. sp. *tritici* or f. sp. *secalis* have been identified and named in barley, *Rpg1*, *Rpg2*, *Rpg3*, *rpg4*, *Rpg5*, *rpgBH,* and *rpg6*. Only *Rpg1*, *rpg4*, and *Rpg5* have been thoroughly investigated at the molecular level. *Rpg2*, identified from Hietpas-5 (CIho 7124) (Patterson et al., 1957); *Rpg3*, from PI382313 (Jedel, 1990); and *rpgBH* from Black Hulless (CIho 666) (Steffenson et al., 1984) do not confer high levels of stem rust resistance and consequently are difficult to assay and have not been mapped (Sun and Steffenson, 2005). The *rpg6* gene was recently identified in a translocation from *Hordeum bulbosum* (Fetch et al., 2009). It confers resistance to *Pgt* race QCCJ, is recessive, and maps to chromosome 6H.

The first stem rust resistance gene identified in barley came from studies with 'Peatland' characterizing a single dominant gene designated *T* (Powers and Hines, 1933), now *Rpg1* for Reaction to *Puccinia graminis* (Sogaard and von Wettstein-Knowles, 1987). *Rpg1* confers durable resistance to a broad range of *Pgt* races but is susceptible to races QCCJ (Sun and Steffenson, 2005) and TTKSK (Ug99) (Steffenson and Jin, 2006). *Rpg1* resistance is considered durable (Steffenson, 1992) because it has successfully controlled stem rust since the release of Kindred barley in 1942 even during the severe epidemics of 1953 and 1954 that devastated the wheat crop (Lejeune, 1951, cited from (Steffenson, 1992). What makes the *Rpg1* gene durable is debatable. Alternative possibilities are that basis of durability resides in the host *R*-gene or perhaps has something to do with the pathogen *Avr* gene. It has been postulated that in some cases the *Avr* gene is so essential for the pathogen's viability that even minor changes in structure can result in loss of virulence. On the other hand, durable *R*-genes are known that have similar structure, for example, *Pto* and *Rpg1* are both protein kinases (Brueggeman et al., 2002; Martin et al., 1993) and durable (Pitblado and Kerr, 1980; Steffenson, 1992). It has been suggested that *R*-genes of the protein kinase class may not evolve rapidly and that their ability

to recognize specific AVR proteins may be highly conserved (Riely and Martin, 2001). Specific expression patterns may also lead to durability, for example, adult plant resistance genes such as *Lr34* and *Yr36* are highly durable (Fu et al., 2009; Krattinger et al., 2009). One also needs to consider other factors such as reduced buildup of inoculum due to a largely resistant wheat crop grown along the spore dispersal corridor.

Kindred barley was developed due to the keen observation powers of farmer Sam Lykken, who during the 1935 stem rust epidemic identified a single rust-free barley plant in his field of 'Wisconsin 37' barley. This single plant selection was released as 'Kindred' in 1942. (We suspect that today this plant genotype would have been patented and farmers would have to pay a handsome premium for the privilege of using it.)

Another source of resistance carrying the *Rpg1* gene was obtained from a 1914 USDA imported bulk landrace from Canton Lucerne in Switzerland. This unimproved landrace gave rise to two sister selections that became 'Chevron' and 'Peatland'. These two cultivars, plus Kindred, have provided essentially all of the stem rust resistance used in barley breeding in the midwestern United States and Canada. With the cloning of the barley *Rpg1* gene, it was shown that all three cultivars possess the same identical allele (Brueggeman et al., 2002) and that the source of Kindred probably came from seed admixtures as proposed earlier (Steffenson, 1992).

In 1989, a new *Pgt* race (QCCJ) with virulence for *Rpg1* was identified (Roelfs et al., 1991) and became one of the most common virulence types in the United States (Roelfs et al., 1993). With the widespread distribution

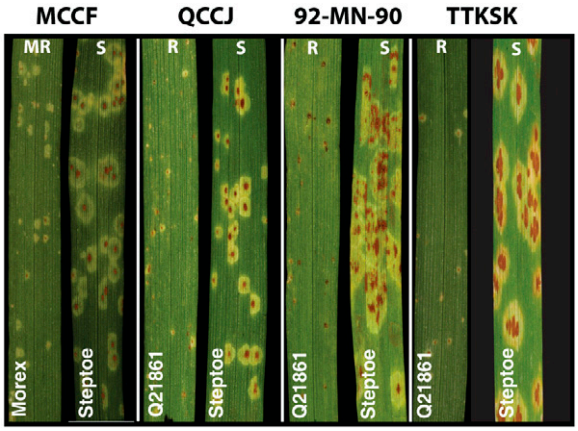

Figure 1. Barley stem rust resistance genes and their reaction to *Puccinia graminis* f. sp. *tritici* races MCCF, QCCJ, and TTKSK and *P. graminis* f. sp. *secalis* isolate 92-MN-90. Race MCCF is avirulent on resistance gene *Rpg1* carried by barley cultivar Morex and line Q21861. Q21861 also carries the *rpg4* and *Rpg5* resistance genes, which, independent of *Rpg1*, also provide resistance to race MCCF (not shown). Races QCCJ and TTKSK are avirulent on the *rpg4/Rpg5* complex but virulent on *Rpg1*. Isolate 92-MN-90 is avirulent on *Rpg5* (*rpg4* not required) but virulent on *Rpg1*. The 'Steptoe' control does not carry any known stem rust resistance genes. M_r, moderately resistant; R, resistant; S, susceptible.

of race QCCJ and the uniform susceptibility of barley grown in the northern Great Plains, severe epidemics were expected but did not materialize. To identify resistance to race QCCJ, over 18,000 barley accessions from the USDA National Small Grains collection were evaluated. The most resistant line identified was Q21861 (Jin et al., 1994). Genetic studies revealed that resistance to race QCCJ in Q21861 is conferred by *rpg4*, a recessive gene. This gene is highly temperature sensitive, being effective at relatively low temperatures (17–22°C), but completely ineffective at temperatures above 27°C (Jin et al., 1994). Q21861 is also resistant to rye stem rust *P. graminis* f. sp. *secalis* (Steffenson, 1992). Rye stem rust resistance in Q21861 is conferred by a single dominant gene designated *Rpg5* (Sun et al., 1996), which is very closely linked to *rpg4* (within 70 kb or about 0.14 cM; Brueggeman et al., 2008) and maps to the long arm of chromosome 5H(7) (Druka et al., 2000). Q21861 is also resistant to several other wheat stem rust races including the new African stem rust race TTKSK (Fig. 1).

The *Rpg1* Gene

Molecular analyses of the barley stem rust resistance genes started with the cloning of the *Rpg1* gene (Brueggeman et al., 2002). We initiated this work with the hope of exploiting rice (*Oryza sativa* L.)–barley synteny to identify the gene. While synteny with rice provided valuable molecular markers and advanced mapping (Kilian et al., 1997), a candidate *Rpg1* gene was not found in the rice chromosome syntenic region (Han et al., 1999).

The *Rpg1* gene was identified by map-based cloning and confirmed by high-resolution genetic and physical mapping, which fortuitously resulted in one recombinant within the gene, and by extensive allele sequencing (Brueggeman et al., 2002). The correct identification of the *Rpg1* gene was also confirmed by stable transformation of 'Golden Promise', known to be lacking detectable *Rpg1* sequences (Horvath et al., 2003). The transformed progeny showed extremely high resistance to *Pgt* race MCCF, which was surprising since the cultivar Morex from which the gene was isolated shows moderate resistance (Fig. 1 and 2).

Analysis of the *Rpg1* mRNA expression showed that it is constitutively expressed at a low level in all plant organs and at all developmental stages except in the leaf epidermis, where it exhibited approximately 30-fold higher expression level than in the whole leaf (Rostoks et al., 2004). Since *Pgt* enters the barley leaf through the stomata, the significance of the elevated *Rpg1* expression in the leaf epidermis cells is not clear. It is possible that the fungus could be detected when it lands or grows along the leaf surface seeking a stomata, but observations made with the fungus on *Rpg1* and *rpg1* near isogenic lines failed to detect any difference up to the point when the fungus penetrated the stomata (B. Steffenson and T. Seeland, unpublished data). Further observations were not possible with live tissue, but sectioning experiments have suggested that resistance appears to be recognized at the haustoria

development stage (Sellam and Wilcoxson, 1976). Experiments with transgenic plants containing variable copy numbers of *Rpg1* and showing variable levels of stem rust resistance showed no correlation between *Rpg1* mRNA levels and resistance phenotype to *Pgt* race MCCF (Horvath et al., 2003). Thus, the elevated level of *Rpg1* mRNA in epidermis cells may be an artifact due to the low complexity of epidermis cell mRNA. *Rpg1* mRNA structural studies identified alternative splice forms, some of which could be translated into full-length proteins (Rostoks et al., 2004). One such alternative splice form appeared to result in a putative trans-membrane domain (discussed below), but we could not confirm this experimentally (Nirmala et al., 2006). Interestingly, the same pattern of expression in the epidermis was observed in a Golden Promise transgenic line carrying a single copy of *Rpg1* gene under control of a 520-bp fragment of its own promoter (Rostoks et al., 2004). In addition, similar alternative splice forms were found in the transgenic line and in Morex.

Rpg1 is a unique gene encoding two tandem kinase domains within a genomic sequence of 4466 bp including 14 exons producing an *in silico* translated protein of 837 aa and 94.5 kDa (Brueggeman et al., 2002) (Fig. 3). Database searches identified homology to an S locus receptor kinase that encodes a plasma membrane-spanning receptor serine–threonine protein kinase presumed to function in self-incompatibility (Takasaki et al., 2000). However, *Rpg1* does not have an obvious receptor domain and *in silico* prediction analysis detected only a weak trans-membrane domain. Thus, the RPG1 protein appears to be a typical kinase except for the unique tandem domains, a motif not previously reported in plant disease resistance genes. The only report of structural and perhaps functional similarity comes from the animal Janus kinases (JAKs), which consist of two tandem kinase domains and function in signal transduction pathways activated by diverse cytokine receptors (O'Shea and Leonard, 1998). There are several members of the JAK family, and their functions are diverse and specific for each gene, but in general they bind to trans-membrane receptors and target gene promoters in the nucleus through the activation of signal transducer and activator of transcription (STAT) transcription factors (Aaronson and Horvath, 2002).

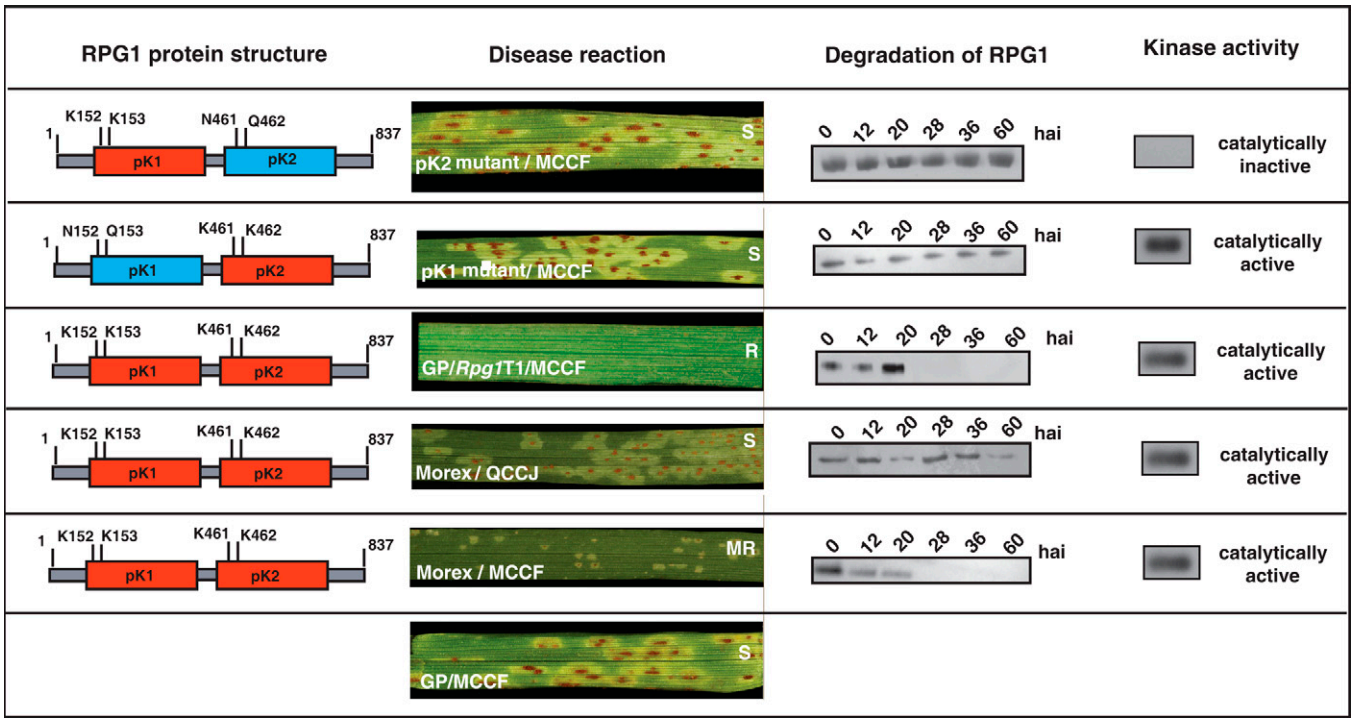

Figure 2. Wild-type and mutant *Rpg1*-mediated reaction to infection with race MCCF and QCCJ. Columns from left to right show cartoon *Rpg1* structure with mutated amino acids indicated; disease reaction phenotype; RPG1 protein response to infection timed in hours; and kinase autocatalytic activity measured in vitro. Lane 1 from top indicates kinase domain 2 mutant with K461 and K462 converted to N and Q, respectively. The resulting mutant transgenic in 'Golden Promise' genomic background is highly susceptible to race MCCF, the RPG1 protein is not degraded in 60 h, and it has no kinase activity. Compare with Lane 2, showing kinase domain 1 mutant with K152 and K153 mutated to N and Q, respectively. This transgenic mutant is also highly susceptible to race MCCF, the RPG1 protein is not degraded in 60 h, but it retains kinase activity, indicating that kinase domain 2 is sufficient for kinase activity, but kinase domain 1 is also required for disease resistance. Compare both with wild-type *Rpg1* transgenic (GP/*Rpg1*T1) in Golden Promise (GP) genomic background (Lane 3) and GP control (Lane 6), which does not have a detectable *Rpg1* gene or protein. RPG1 protein in GP/*Rpg1*T1 is degraded 20 to 28 h after infection, indicating that protein degradation is associated with disease resistance. Autocatalytic kinase activity is present. 'Morex' (Lanes 4 and 5) with wild-type *Rpg1* shows resistance to race MCCF but susceptibility to race QCCJ. The RPG1 protein is degraded between 20 and 28 h in MCCF infection but not with QCCJ infection, indicating that RPG1 degradation is a specific response to infection with avirulent, but virulent races. Autocatalytic activity is retained in both cases.

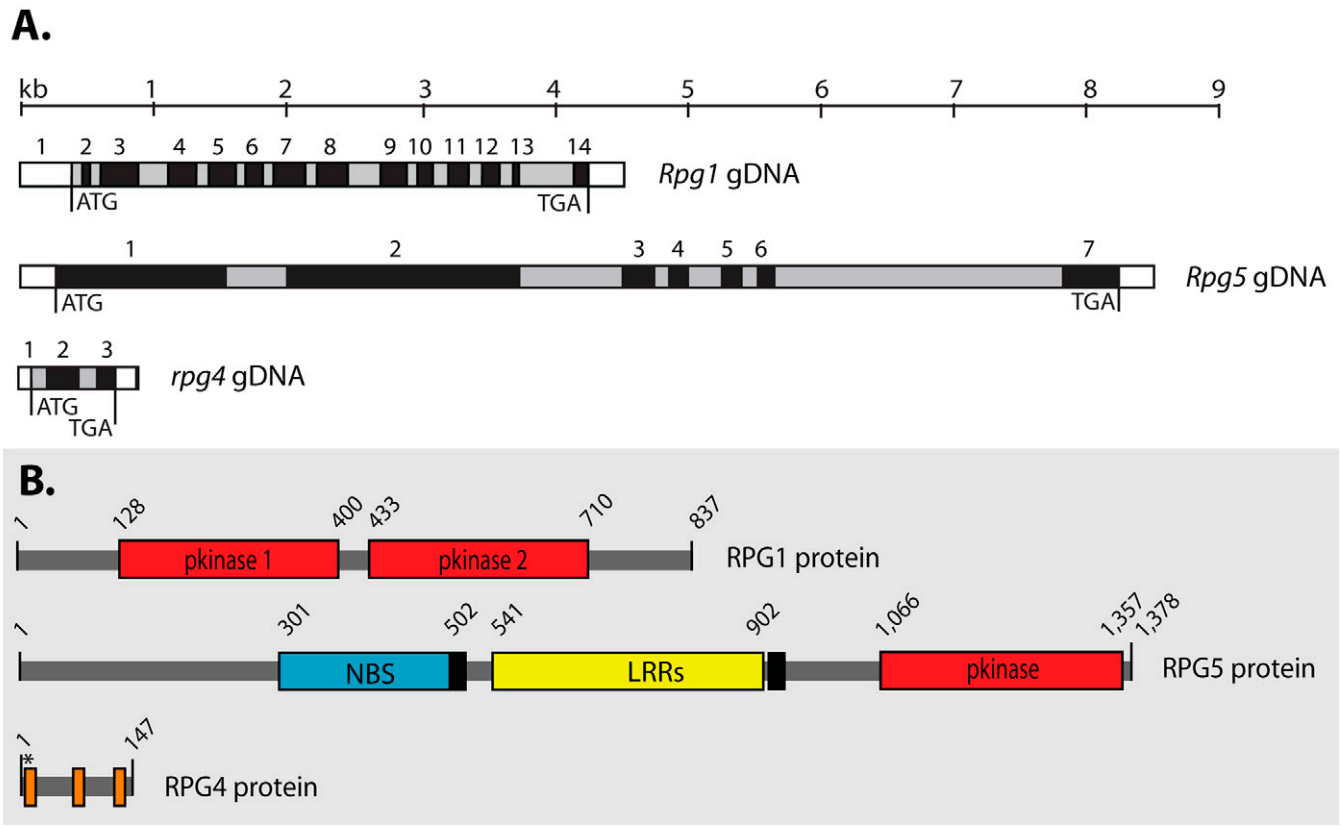

Figure 3. Stem rust resistance gene organization and predicted protein structures. (A) Genomic DNA organization of *Rpg1*, *Rpg5*, and *rpg4* genes (labeled to the right) with exons (black) introns (gray), 5 prime, and 3 prime untranslated regions (white). Exons are numbered above. ATG represents the start methionine codon and TGA represents the stop codon. The scale shown above is in kilobases. (B) Protein domain structures for RPG1, RPG5, and RPG4 are shown to scale with predicted boundaries above. pkinase denotes serine–threonine protein kinase domains, NBS denote nucleotide binding site, LRRs denote leucine rich repeats, and orange bars indicate position of conserved amino acids at actin binding sites. Asterisk indicates the conserved putative regulatory phosphorylation site. Proteins are labeled to the right.

The animal JAKs are unique and no doubt different from *Rpg1*; nevertheless, this well-investigated system provides us with a model for *Rpg1* function. Analysis of the RPG1 protein identified that, as with the JAKs, only one of the kinase domains (pK2) was an active kinase while the pK1 domain was inactive even though required for resistance (Fig. 2) (Nirmala et al., 2006). It was also determined that the RPG1 protein is mostly cytoplasmic with a small but significant amount associated with membranes. This situation is somewhat similar to what was found with JAKs, which were originally reported to be cytosolic, but recently shown to be associated with the plasma membrane and endoplasmic reticulum (Behrmann et al., 2004). We do not know what the role is, if any, of the membrane associated RPG1 protein, but it is possible that like the JAKs, RPG1 associates with the membrane through a membrane bound receptor and this association is either weak or it is induced by some signal from the pathogen. We have not examined RPG1 location under pathogen attack conditions, but surprisingly, infection resulted in rapid degradation of the RPG1 protein, but only by avirulent and not by virulent pathotypes (Fig. 2) (Nirmala et al., 2007). Furthermore, this degradation takes place through the ubiquitin–proteasome pathway (Nirmala et al., 2007) similar to degradation of JAK2, which has also been shown to occur via the ubiquitin–proteasome pathway (Ungureanu et al., 2002).

The role of the pseudokinase (pK1) domain in RPG1 function is unknown. Substituting the two adjacent lysines K152 and K153 of pK1 with asparagine (N) and glutamine (Q) did not inactivate the RPG1 protein kinase autocatalytic activity, but it did result in a susceptible disease reaction of the stable pK1 mutant transgenic line (Fig. 2) (Nirmala et al., 2006). The wild-type RPG1 interacts with several different proteins in the yeast two-hybrid system, but the double KK pseudokinase mutant does not (Nirmala et al., unpublished data). However, when the two lysines were mutated individually, K152N and K153Q, both proteins interacted the same as wild-type RPG1. These data suggested that there may be some structural constraints on the pseudokinase domain that were induced when the side-by-side lysines were mutated together, but not individually. Deletion of the RPG1 pseudokinase domain abolished its ability to interact with the proteins identified as interacting with the wild-type RPG1 (see below), suggesting possible involvement of the pseudokinase domain in protein conformation and protein–protein interactions.

Two barley cDNA libraries, one from uninfected and one from *Pgt* race MCCF infected Morex leaves, were screened in the yeast two-hybrid system with RPG1 as the bait (Nirmala et al., unpublished data). The libraries consisted of 2×10^6 and 4.2×10^6 transformants and collectively yielded 38 putative positive clones through two rounds of selection. The clones identified to date include *HvPti1*, *HvPti4*, *5*, and *6*, a Map3 kinase, *HvRin4*, *HvHSP17*, a Myb-like transcription factor, *HvEdr1*, and extensin-like protein. As mentioned above, all of these proteins interact with wild-type RPG1, but none of them interact with the pseudokinase double mutant KK152,153NQ, the kinase double mutant KK461,462NQ, the kinase single mutant K461N (which eliminates the autocatalytic kinase activity), or the pseudokinase deletion. The pseudokinase single mutants K152N and K153Q are still fully interactive as is the kinase mutant K462Q, which is not involved in the kinase catalytic site (Nirmala et al., unpublished data). The role of each of these interactors is being further characterized.

The JAK pseudokinase (JH2) domain carries out a number of functions that may be relevant to the *Rpg1* pseudokinase domain function. Although catalytically inactive, it appears to be a negative and positive regulator of the JAK kinase (JH1) domain. One of the functions attributed to the JH2 domain is negative regulation of the JH1 domain activity. Deletions of JH2 resulted in activation of the JH1 domain (Saharinen and Silvennoinen, 2002) and mutation in *Drosophila* JH2 JAK was found to up-regulate kinase activity and result in leukemia (Luo et al., 1997). This regulation may be performed via a physical interaction between the JH1 and JH2 domains (Chen et al., 2000).

Possible RPG1 interactors have been identified with the yeast two-hybrid system as described above and by mutant, microarray, and expression quantitative trait loci (eQTL) analyses. Screening of fast neutron induced mutants identified a gene (designated *rpr1*, required for Puccinia resistance) that is required for *Rpg1* function (Zhang et al., 2006). The candidate *Rpr1* gene was identified as encoding a serine–threonine protein kinase by transcript-based cloning (Zhang, 2006). However, the role of this kinase in *Rpg1*-mediated stem rust resistance remains elusive. It may function downstream of *Rpg1*-mediated response, but it does not interact directly with the RPG1 protein in the yeast two-hybrid assay (Zhang, 2006). A parallel gene expression profiling experiment using the Barley1 microarray was conducted in two isolines, cultivar Golden Promise and its single copy *Rpg1* transgenic line, across six time points. Comparisons were done in all-pairwise combinations using the transgenic line (G02-448F-3R) and cultivar Golden Promise as well as the *Pgt* races MCCF (avirulent on *Rpg1*) and QCCJ (virulent on *Rpg1*). A total of 34 probe sets exhibited expression pattern differences between the susceptible (Golden Promise) and resistant (*Rpg1* transgenic) lines with the *Pgt*-MCCF treatment, while 14 probe sets exhibited expression pattern differences between *Pgt* MCCF (avirulent) and *Pgt* QCCJ (virulent) inoculated *Rpg1* transgenic Golden Promise (Zhang et al., 2008). Such microarray analysis identified possible candidate genes for signaling pathway(s) toward disease resistance but did not differentiate causal from reactive genes.

Usually, barley–stem rust fungus interaction phenotypes are scored semiquantitatively but mapped as Mendelian traits, thus accounting only for major genes. However, trait residual variance, if it is genetically determined, can identify additional loci responsible for the trait variation. We investigated the full potential of semiquantitative scoring by mapping barley and *Pgt* interaction phenotypes in 'Steptoe' (susceptible) × Morex (resistant) segregating population as quantitative traits (Druka et al., 2008). This analysis resulted in the identification of six loci. Candidate genes underlying these loci were identified by correlating interaction trait values with mRNA abundance values of about 20,000 barley genes that were also measured and mapped in the Steptoe × Morex DH population as eQTLs (Druka et al., 2008). The strongest quantitative trait locus (QTL) was identified at the *Rpg1* locus on chromosome 7H(1), as expected. It coincided with the strong *Rpg1* eQTL. This was a surprising result, given that *Rpg1* mRNA accumulation level was low—close to the Barley1 microarray detection threshold level in both parents and in the recombinant DH lines. Another strong eQTL was found on chromosome 2H. Unfortunately, correlation analysis revealed hundreds of possible candidate genes, making it impossible to narrow the field to just a few. However, it did include a putative sensory transduction histidine kinase, which was also previously identified as strongly downregulated in the *Rpg1* suppressor mutant *rpr1* (Zhang et al., 2006). Thus, identification of a sensory transduction histidine kinase as possibly involved in barley–stem rust interactions makes it a strong candidate for further investigations. Curiously, resistance QTL were not detected at the *Rpr1* locus on chromosome 4H. This could be due to lack of *Rpr1* expression polymorphism in the Steptoe × Morex population. Additional QTL were identified on chromosomes 3H, two on 5H(7) and another one on 7H(1). The QTL on 3H, 7H(1), and one on 5H(7) coincided with the heat shock protein (*Hsp17*) gene family eQTL, among others. The HSP17 protein may be relevant since it was also identified as an RPG1 interactor in the yeast two-hybrid assay (see above). The last and barely significant QTL was identified on chromosome 5H(7) in the *rpg4/Rpg5* stem rust resistance gene locus region. This was surprising since the *rpg4/Rpg5* stem rust resistance genes are not polymorphic in the Steptoe × Morex population but were identified in a different cross (Steffenson et al., 1995). Nevertheless, these data suggest possible interaction between the *Rpg1* and *rpg4/Rpg5* loci, which was not previously detected.

The *rpg4/Rpg5* Genes

Initial mapping of the *rpg4* locus was done using RAPD and RFLP markers and positioned the gene to the long arm of chromosome 5H(7) (Borokova et al., 1995).

High-resolution genetic and physical mapping provided localization of the *rpg4/Rpg5* locus to a small region of chromosome 5H(7) (Druka et al., 2000; Han et al., 1997; Kilian et al., 1997). However, substantial additional genetic and physical mapping was required for isolation and confirmation of the *rpg4/Rpg5* genes (Brueggeman et al., 2008). A 70-kb region between markers ARD5016 and ARD5112 was cloned, sequenced, and shown to contain five candidate genes, two encoding predicted *R*-gene like NBS-LRR proteins designated HvRGA1 and HvRGA2, two actin depolymerizing factors designated HvADF2 and HvADF3, and a protein phosphatase 2C-like protein (HvPP2C) (Fig. 4). Since the BAC clones and sequence was derived from the susceptible cultivar Morex, the equivalent sequence from resistant line Q21861 was obtained, which resulted in the surprising discovery that HvPP2C gene was missing and that a S/TPK gene had been inserted adjacent to the HvRGA2 gene. All of the other genes were present and essentially identical to those found in Morex. On the basis of additional allele sequencing and analysis, HvRGA2 was identified as the *Rpg5* gene. Identity of the *Rpg5* gene was also confirmed by VIGS analysis (Brueggeman et al., 2008). Comparison of cDNA and gDNA sequences identified seven exons in total genomic sequence of 8504 bp encoding a 4.4-kb mRNA, which on *in silico* translation resulted in a 1378 aa (151.6-kDa) protein (Fig. 3). The large mRNA encodes the NBS-LRR and S/TPK domains in a single transcript, a unique gene structure previously unknown among plant disease resistance genes.

Recombinant analysis confined the *rpg4* gene to a 1-kb interval encoding only the HvADF2 protein. Although the genetic evidence is very strong, to date we have not obtained independent evidence that the *Adf2* gene is in fact *rpg4*; thus it remains a highly probable candidate. *HvAdf2* (*rpg4*) encodes an apparently functional *Adf* gene containing three exons in a total sequence of 906 bp coding for a 147 aa (16.17-kDa) protein (Fig. 3). Even though ADF proteins are important in cytoskeleton rearrangements and have been reported to be involved with nonhost and basal resistance (Kobayashi and Kobayashi, 2007; Kobayashi et al., 1997; Miklis et al., 2007), the role of the ADF2 protein in resistance to a specific stem rust race is unique. Current evidence suggests that the *rpg4* gene does not act independently but rather that it is dependent on the *Rpg5* gene for function (Brueggeman et al., 2009). In fact, the *Rpg5* gene appears to be required for resistance to stem rust races MCCF and TTKSK in addition to QCCJ. Since the *rpg4* gene is genetically recessive and temperature dependent, it may have a negative function on the ability for the fungus to establish itself within the plant. One hypothesis is that the fungus captures the wild-type ADF2 protein function to facilitate feeding itself via the cytoskeleton and that the *rpg4* encoded ADF2 is unable to respond. However, we know that the *rpg4* gene by itself is not sufficient to confer resistance, perhaps due to failure to detect the fungus (Brueggeman et al., 2009). The temperature sensitivity may suggest that other or additional hypotheses are needed. The classic interpretation of temperature sensitivity is that the protein structure is stable and functional at the lower temperature, but may become unstable at higher temperatures. This interpretation would mean that the *rpg4* gene is transcribed and translated and the protein has some positive function at the lower temperature. We know that *rpg4* is transcribed but do not have data about the protein yet.

Evolution

The cloning and characterization of the barley stem rust resistance genes identified unique plant disease resistance genes. The dual putative kinase domain structure of *Rpg1* has not been previously observed in disease resistance genes and is in fact rare in the completely sequenced plant genomes like rice and *Brachypodium*. The only similar model is the animal JAK (Aaronson and Horvath, 2002). Analysis of Morex *Rpg1* family members identified five additional genes with variable homology, but only three had the dual kinase domain structure (Brueggeman et al., 2006). Of these, ABC1037 is the most similar and also closely linked to *Rpg1* (ca. 50 kb), suggesting an origin by tandem duplication. It is expressed at the mRNA level but diverges on the 3′ end and has no known function. ABC1036 and ABC1040 are similar to one another and closely linked on chromosome 5H(7) but significantly diverged from *Rpg1*, particularly in the kinase 2 domain. The presence of three of these tandem kinase domain genes and their scattered location in the Morex genome suggest that this particular structure evolved some time ago. Similar *Rpg1* homologs with tandem kinase domain structure were also observed in wheat, confirming an ancient origin.

Similarly, the *Rpg5* gene is unique in combining the three common disease resistance motifs into a single gene. A search of the rice and *Brachypodium* genomes did not identify a gene with similar structure. Separate NBS-LRR and S/TPK genes with homology to the domains in *Rpg5* were found, but on different chromosomes or supercontigs in rice and *Brachypodium*, respectively (Drader et al., personal communication, 2009). Even in closely related wheat the NBS-LRR and S/TPK homologous domains appear to be encoded by separate genes (Brueggeman et al., 2009).

To gain a better understanding about the origins of these genes, we analyzed their sequences from a variety of wild barley (*H. vulgare* ssp. *spontaneum*) accessions. Sequence analysis of *Rpg1* from resistant North American barley cultivars revealed no amino acid polymorphism. This suggested that there was a single source for *Rpg1* in the North American germplasm, and it probably came from the unimproved bulked seed lot obtained by the USDA from Switzerland in 1914. Therefore, additional land races from Switzerland were included in the analysis.

A set of about 100 *H. vulgare* ssp. *spontaneum* accessions was tested with *Pgt* races HKHJ and MCCF. Race HKHJ has been previously shown to be specific for

Rpg1-mediated stem rust resistance, while race MCCF is known to be avirulent for additional stem rust resistance genes aside from *Rpg1* (Sun and Steffenson, 2005). Since no *H. vulgare* ssp. *spontaneum* accessions and only one Swiss landrace exhibited resistance to race HKHJ, four additional lines most resistant to race MCCF and an equal number of susceptible lines from each group were selected for sequencing. The Swiss landrace resistant to race HKHJ proved to have an *Rpg1* gene identical to that previously sequenced from North American barley cultivars (Mirlohi et al., 2008). Thus, it is most likely from the same source as the one imported into the United States in 1914 even though its collection site in Canton Graubunden was quite distant from the Canton Lucerne. None of the other lines had functional *Rpg1* genes. These data indicate that a functional *Rpg1* gene is very rare in *H. vulgare* ssp. *spontaneum* and Swiss land race populations. A curious GTT insertion was present in seven out of the eight accessions containing a defective *Rpg1*-like gene and thus is very common. This insertion results in an S to R substitution at position 319 and an F insertion at position 320 leading to susceptibility to both races HKHJ and MCCF. We do not know if the *Rpg1* gene with a GTT insertion carries out some other function or confers resistance to ancient *Pgt* races, but its occurrence and persistence in a diverse population suggest that some function was likely. On the other hand, the absence of a functional *Rpg1* gene in the populations tested suggests that the GTT insertion may be a recent evolutionary event. The populations tested are admittedly small, but the same lines tested for putative *Rpg5* presence using the *Pgs* isolate 92-MN-90 showed frequent presence of an apparently functional *Rpg5* gene and significant allele variation (Brueggeman et al., unpublished data). Future work will require much more extensive testing of the *H. vulgare* ssp. *spontaneum* populations.

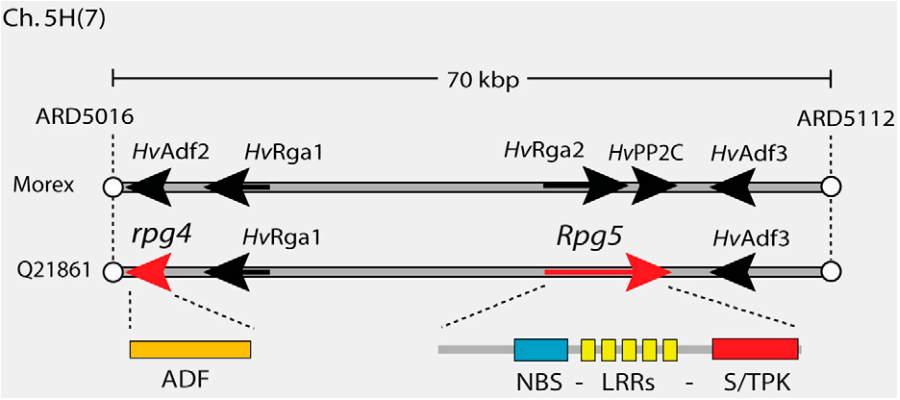

Figure 4. Sequence annotations of the *rpg4/Rpg5* genomic region and protein structures. Gray horizontal bars represent sequenced regions from barley cultivar Morex (susceptible) and line Q21861 (resistant) labeled on the left. White circles represents position of the flanking genetic markers, ARD5016 and ARD5112 labeled above. Scale is shown above in kilobases. The red arrows represent the *rpg4* (*Adf2*) and *Rpg5* (NBS-LRR-S/TPK) stem rust resistance genes. Black arrows indicate other annotated genes. Cartoon protein domain structures of RPG4 and RPG5 are shown below the Q21861 sequence annotation.

Future Research and Prospects

Stem rust is a serious disease that has caused significant yield losses in barley and wheat in the past. However, over the past 60 years in the United States, the disease has been controlled by the strategic use of disease resistance genes. Nevertheless, due to the pathogen's high potential for variability by mutation and sexual or asexual recombination and its ability to reproduce rapidly and spread over great distances, it remains a serious threat that cannot be ignored in the short or long term. This was painfully illustrated by the recent emergence of TTKSK, a widely virulent race of stem rust that was first isolated in Uganda but has since spread throughout East Africa and is now in the Middle East (Nazari et al., 2009). Race TTKSK is predicted to spread to the world's most important wheat and barley growing regions in the near future (Singh et al., 2008; CIMMYT, 2007).

Stem rust resistance research is limited to the crops that are infected by it, barley, wheat, rye, and oat. Rice is apparently immune to stem rust. It would be intriguing to exploit rice's immunity to rust for use in the small grain cereals that are congenial hosts for *P. graminis*, but this may be a difficult trait to dissect. Moreover, this type of immunity is most likely due to nonhost resistance and/or to the environment where it is grown. In either case, it is not likely to be transferred to wheat and barley. Thus, barley, being a true diploid compared to the closely related hexaploid wheat, is the best model in which to study host–pathogen interactions with the expectation that knowledge will lead to effective control measures. Barley may not be a perfect model, but it is quite tractable. The arguments against barley as a model, such as its large genome, are not applicable in the age of rapid and inexpensive sequencing that promises to become even more rapid and less expensive in the near future. The one limitation to the barley model system that is yet to be overcome is transformation difficulty. However, it is possible, and future research should result in improvements.

Host–pathogen interactions are complex and seem to raise more questions than answers. Our situation is similar, and a number of questions remain to be answered. We will not go into details here of the numerous experiments that need to be done but rather focus on what we consider to be the important questions. First and foremost: What is it that makes *Rpg1*-mediated stem rust resistance so widely effective against different pathogen races leading to its durability? Durability is highly desired by plant breeders because it allows them to focus on problems other than continuously breeding for resistance against new races of the pathogen. Unfortunately,

most stem rust resistance genes are not durable, and in wheat this led to repeated boom-and-bust cycles until gene pyramiding was instituted. In contrast, the barley *Rpg1* gene has provided resistance to stem rust races in the midwestern United States and Canada since the early 1940s when the first cultivar with the gene was released. We know that wheat has genes similar to *Rpg1*, but do they function in resistance to wheat stem rust races or do they function at all? If not, then why and how can they be modified to function? To answer those questions, we first need to understand what makes the *Rpg1* gene durable. The *Rpg1* gene does not provide resistance to the new TTKSK threat, but *Rpg5* does. How does *Rpg5* work, and is there a wheat equivalent? We know that the *Adf2* gene is required to interact with *Rpg5* to confer resistance to race TTKSK. Plant genomes have multiple *Adf* genes. Analysis of *Arabidopsis* and rice sequences identified 12 ADF-like proteins encoded by each genome (Feng et al., 2006). Is *Adf2* unique, and if so, what makes it unique and how does it work? Answers to these questions may reveal some clues as to how to control race TTKSK before it seriously damages the world's wheat and barley crops.

These few examples illustrate that much work remains to be done and it needs to be done urgently. Stem rust can and will seriously damage the wheat and barley crop if not vigorously combated. The best way to defend against stem rust is by deploying effective resistance genes. To deploy those genes, we need to understand how they work.

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